# The antihypertensive drug pindolol attenuates long-term but not short-term binge-like ethanol consumption in mice

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## Abstract

Alcohol dependence is a debilitating disorder with current therapies displaying limited efficacy and/or compliance. Consequently, there is a critical need for improved pharmacotherapeutic strategies to manage alcohol use disorders (AUDs). Previous studies have shown that the development of alcohol dependence involves repeated cycles of binge-like ethanol intake and abstinence. Therefore, we used a model of binge–ethanol consumption (drinking-in-the-dark) in mice to test the effects of compounds known to modify the activity of neurotransmitters implicated in alcohol addiction. From this, we have identified the FDA-approved antihypertensive drug pindolol, as a potential candidate for the management of AUDs. We show that the efficacy of pindolol to reduce ethanol consumption is enhanced following long-term (12 weeks) binge–ethanol intake, compared with short-term (4 weeks) intake. Furthermore, pindolol had no effect on locomotor activity or consumption of the natural reward sucrose. Because pindolol acts as a dual beta-adrenergic antagonist and 5-$HT_{1A/1B}$ partial agonist, we examined its effect on spontaneous synaptic activity in the basolateral amygdala (BLA), a brain region densely innervated by serotonin and norepinephrine-containing fibres. Pindolol increased spontaneous excitatory post-synaptic current frequency of BLA principal neurons from long-term ethanol-consuming mice but not naïve mice. Additionally, this effect was blocked by the 5-$HT_{1A/1B}$ receptor antagonist methiothepin, suggesting that altered serotonergic activity in the BLA may contribute to the efficacy of pindolol to reduce ethanol intake following long-term exposure. Although further mechanistic investigations are required, this study demonstrates the potential of pindolol as a new treatment option for AUDs that can be fast-tracked into human clinical studies.

## Introduction

Alcohol addiction is a debilitating disorder that accounts for nearly 3.8 percent of global deaths and 4.6 percent of disability adjusted life years (Rehm *et al.* 2009). Despite concerted efforts, there are only three FDA-approved medications to treat alcohol use disorders (AUDs), namely acamprosate, naltrexone and disulfiram, all of which have limited or modest efficacy (Anton *et al.* 2006; Mann *et al.* 2013).

The development of alcohol addiction is progressive and often occurs over an extended period of time. Initially, alcohol consumption causes acute changes in the brain, which leads to the development of tolerance (Gilpin & Koob 2008). This results in an escalation of consumption that can manifest as episodes of binge-like alcohol intake. Binge–consumption of alcohol is a commonly observed trait in human alcohol dependence (Robin *et al.* 1998).

Evidence from animal studies also suggests that repeated cycles of binge–ethanol consumption and abstinence contribute to the development of alcohol dependence (Hwa *et al.* 2011). Furthermore, changes in signalling from structures of the extended amygdala cause sensitization to the negative symptoms of alcohol withdrawal and are thought to enhance craving for alcohol during abstinence. In particular, these neuroadaptations increase the activity of neurotransmitters involved in stress such as corticotrophin-releasing factor (Gilpin & Koob 2008). Additionally, changes in the function and signalling of other neurotransmitters including norepinephrine (NE) and serotonin (5-HT) have also been implicated in negative withdrawal states of alcohol addiction (Fahlke *et al.* 2012; George 1994; Gilpin & Koob 2010). Evidence suggests that alcohol-induced changes in brain NE activity may influence motivation to drink alcohol (Shirao *et al.* 1988) in dependent models (Gilpin & Koob 2010). Additionally, increased noradrenergic activity has been associated with stress disorders such as post-traumatic stress disorder (Berardis *et al.* 2015). The use of drugs including propranolol and prazosin, which inhibit NE activity, have been shown to reduce ethanol consumption in alcohol-dependent animals (Gilpin & Koob 2010; Walker *et al.* 2008). Moreover, depletion of NE synthesis in studies with β-hydroxylase knockout mice has been shown to reduce ethanol preference and consumption (Weinshenker *et al.* 2000).

Like NE, dysregulation of serotonergic signalling is also implicated in the enhancement of negative emotional states such as anxiety, which contribute to alcohol seeking behaviour (Halliday, Baker, & Harper 1995; LeMarquand, Pihl, & Benkelfat 1994; McBride *et al.* 1989). Acute alcohol consumption increases 5-HT levels in mesolimbic reward regions of the brain including the nucleus accumbens (NAc) (LeMarquand, Pihl, & Benkelfat 1994). However, following multiple ethanol exposures, 5-HT levels in the NAc are no longer elevated but are rapidly decreased during withdrawal. These levels are restored by alcohol re-exposure (De Montis *et al.* 2004; Weiss *et al.* 1996), suggesting that changes in 5-HT levels may contribute to alcohol-seeking behaviour with research supporting the roles of 5-$HT_{1A}$ and 5-$HT_{1B}$ receptors subtypes in the development of alcohol dependence (Miczek, Hussain, & Faccidomo 1998; Sari 2013).

Because both of these neurotransmitters appear to modulate alcohol-drinking behaviours, we tested the efficacy of a series of compounds with activity at noradrenergic and 5-HT receptors, on short-term and long-term binge-like consumption of ethanol using the drinking-in-the-dark (DID) paradigm in mice (Rhodes *et al.* 2005). The DID paradigm is a robust protocol used to achieve high levels of ethanol consumption in non-ethanol-dependant mouse strains. High alcohol-drinking mouse strains including C57BL/6J, which have been used in this study, typically achieve mean blood ethanol concentrations of more than 100 mg/ml using this paradigm and exhibit binge patterns of ethanol consumption, which result in symptoms of behavioural intoxication (Thiele & Navarro 2014). The most salient feature of this model as a screening tool for potential pharmacological compounds to manage AUDs is the maintenance of binge ethanol consumption over extended periods (Thiele & Navarro 2014). Because alcohol dependence develops following prolonged excessive alcohol consumption, we allowed adolescent mice to self-administer ethanol using the DID paradigm for 12 weeks. We also determined the effect of the test compounds on short-term ethanol consumption (4 weeks) and consumption of the natural reward sucrose to assess their validity as potential candidates for the treatment of AUDs in humans.

From these experiments, we have identified pindolol, an antihypertensive drug with dual pharmacological activity at both β-adrenergic receptors (βAR) and 5-$HT_{1A/1B}$ receptors, as a potential new candidate to treat AUDs. We show that pindolol produces robust decreases in

ethanol consumption in C57BL/6J mice following long-term but not short-term exposure. Additionally, pindolol displayed selectivity for ethanol over a natural reward, having no effect on sucrose consumption following long-term intake. Because pindolol exhibits complex pharmacology, acting as a non-selective antagonist at $\beta_1$-AR and $\beta_2$AR and a partial agonist at the low-affinity site of the $\beta_1$AR ($\beta_{1L}$-AR), $\beta_3$AR (Kaumann & Molenaar 2008) and 5-$HT_{1A/1B}$ receptors (Skelin, Fikre-Merid, & Diksic 2012), we tested the efficacy of additional compounds with activity at these receptors using the long-term model. We show that (-)-CGP12177, a compound closely mimicking the pharmacology of pindolol at βARs, caused a trend towards a reduction in ethanol consumption (not significant) at the highest dose tested. Furthermore, the 5-$HT_{1A}$ partial agonist buspirone significantly reduced both long-term ethanol and sucrose consumption.

Because the BLA contains high expression levels of the receptor targets of pindolol (βARs and 5-$HT_{1A/1B}$ receptors; Butler, Chappell, & Weiner 2014; Silberman, Ariwodola, & Weiner 2012), we investigated the effect on spontaneous synaptic transmission in this brain region and found that pindolol (10 μM), significantly decreased the frequency of spontaneous excitatory post-synaptic currents in BLA principal neurons in naïve animals but increased the frequency of spontaneous excitatory post-synaptic currents in BLA principal neurons in long-term ethanol consuming mice. These experiments demonstrate an adaptive change of pindolol's molecular targets, which may contribute to its efficacy in reducing ethanol consumption following repeated, long-term exposure.

We believe that the highly selective effect of pindolol to reduce ethanol consumption following long-term binge-like intake demonstrates a novel treatment method for AUDs that is immediately available for advancement into human studies.

# Materials and Methods

## Animals and housing

Male C57BL/6J mice (5-week old, ARC, WA, Australia) were individually housed in ventilated Plexiglas® cages with *ad libitum* access to food and water in a climate-controlled, reverse 12-hour light/dark cycle room (lights off at 9:00 AM). Mice were given 1 week to acclimatize to the housing conditions and handling, prior to the start of experiments. All procedures were pre-approved by the Queensland University of Technology Animal Ethics Committee and the University of Queensland Animal Ethics Committee.

## Drinking-in-the-dark paradigm

To evaluate the baseline consumption, we used the DID model of ethanol consumption (Rhodes *et al.* 2005). Briefly, mice were individually housed in double-grommet cages and given access to one bottle of 20 percent (v/v) ethanol and one bottle of filtered water for a 2-hour period (12 to 2 PM), 3 hours into the dark cycle, Monday to Friday. The ethanol-containing and water-containing bottle sides were switched every presentation to prevent side preference. Two bottles of filtered water were available at all other times. All fluids were presented in 50 ml, graduated, plastic centrifuge tubes (Corning Centristar, NY, USA) fitted with rubber stoppers and a 2.5 inch stainless-steel sipper tube with double ball bearings. Bottles were weighed prior to and at 30 minutes and 2 hours after presentation, and measurements were taken to the nearest 0.1 g. We also measured 5 percent sucrose

consumption using the DID paradigm where the mice were given intermittent access to one bottle of 5 percent (v/v) sucrose instead of ethanol. Mouse weights were measured daily to calculate the adjusted gram per kilogram intake.

## Drug testing

Once stable baseline drinking levels of 20 percent (v/v) ethanol or 5 percent sucrose were established, we evaluated the acute effects of pindolol administration on short-term (vehicle, 8, 16 and 32 mg/kg, i.p.) and long-term (vehicle, 4, 8, 16 and 32 mg/kg, i.p.) ethanol intake. For long-term ethanol drinking mice, pindolol was administered in two cohorts of mice that either received pindolol (vehicle, 8, 16 and 32 mg/kg, i.p.) or pindolol (vehicle, 2, 4 and 8 mg/kg, i.p.). Additionally, we tested the effect of pindolol on short-term and long-term sucrose consumption (vehicle, 8, 16 and 32 mg/kg, i.p.). We also tested buspirone hydrochloride (vehicle, 1, 2.5 and 5 mg/kg, i.p.) and (-)-CGP12177 (vehicle, 30, 50 and 80 mg/kg, s.c.) in long-term ethanol consuming animals and buspirone hydrochloride (vehicle, 1, 2.5 and 5 mg/kg, i.p.) in long-term sucrose drinking animals. All drugs were prepared on the day of the experiment and administered to mice in a volume of 10 µl/gm, 30 minutes before presentation of the bottles. Each injection was given seven days apart using a Latin square design; thus, each animal served as its own control.

## General locomotor activity

Locomotor studies were run in an open field locomotor chamber (40 × 40 cm) using the ANY-maze behaviour tracking software (Stoelting, Wood-dale, IL). The study was run in four daily 2-hour sessions. After habituation to the boxes (Day 1) and injections (Days 2 and 3) testing was conducted on day 4. The animals were assigned to one of two treatment groups. One group received buspirone hydrochloride (2.5 or 5 mg/kg, i.p.) or vehicle and the other group received pindolol (32 mg/kg), ($n = 6$ per group). After 60 minutes, a single injection of the assigned treatment was given and subsequently the session continued for an additional 60 minutes. Data were collected across the entire 2-hour session and recorded as distance travelled in metres.

## Loss of righting reflex

A single intraperitoneal injection (i.p.) of 3.2 g/kg (20 percent v/v) EtOH was administered to assess the effect of pindolol on ethanol sensitivity. The latency to loss of righting reflex (LORR) was recorded as the time from EtOH injection to the time the mouse was unable to right itself 3 times within a 15-second period from the supine position. LORR was recorded as the time elapsed from when the mouse was unable to right itself 3 times within a 15-second period from the supine position until it recovered and was able to right itself 3 times within a 15-second period. C57BL/6J mice were divided into two groups that received either vehicle or pindolol (32 mg/kg, i.p.), 30 minutes before ethanol was administered.

## Electrophysiology

Slice preparation: Six-week-old male C57BL/6J or 18-week-old male C57BL/6J ethanol-consuming mice were deeply anaesthetized with intraperitoneal sodium pentobarbitone (~80 mg/kg; Vetcare, Brisbane, Australia), then decapitated and brain was removed, fixed using cyanoacrylate glue to a holder in a metal bath and submerged in ice cold high $Mg^{2+}$

Ringer solution that contained (in mM): 130 NaCl, 3 KCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 5 $MgCl_2$, 1 $CaCl_2$ and 10 D-glucose (osmolarity~300 mOsm, Vapro 5520 osmometer, Wescor, South Logan, UT) (Bellingham & Berger 1996). Coronal slices of 300 μm containing the BLA were transferred from the ice-cold bath solution (high $Mg^{2+}$) into fresh solution warmed to 34°C and incubated for 60 minutes. The sections were then transferred to normal Ringer (as previously mentioned except, 1 mM $MgCl_2$ and 2 mM $CaCl_2$) solution and incubated for at least 45 minutes at room temperature (22–24°C) prior to recording. All solutions were continuously bubbled with 95 percent $O_2$/5 percent $CO_2$.

We recorded from BLA principal neurons using electrodes pulled from borosilicate glass capillaries (Vitrex Modulohm, Edwards Medical, NSW, Australia) filled with an internal solution containing (in mM): 135 mM $Cs^+$-methanesulphonate ($Cs^+MeSO_4$), 6 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 5 mM Na-HEPES, 3 mM ATP-$Mg^{2+}$ and 0.3 mM GTP-Tris (pH 7.25 with KOH, osmolarity of 305 ± 5 mOsm) (Kanjhan & Bellingham 2013). Slices were continuously superfused (1–2 ml/min) with normal Ringer solution at room temperature (22–24°C). BLA principal neurons (large piriform shaped soma and high cell capacitance) were visualized on a Nikon E600FN (Tokyo, Japan) microscope fitted with IR-DIC optics. Whole cell voltage-clamp recordings were made with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA). Data were acquired at a sampling rate of 10 kHz, low-pass filtered at 2 kHz and stored on a Windows computer using PClamp 10.2 software and a Digidata 1332A digitizer (Axon Instruments). To record spontaneous inhibitory post-synaptic currents (sIPSCs), BLA principal neurons were voltage clamped at a holding potential of 0 mV, and currents were pharmacologically isolated by addition of DL-2-amino-5-phosphonovaleric acid (APV) (50 μM; Sigma-Aldrich, NSW, Australia) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μM; Sigma-Aldrich, NSW, Australia) to the bath solution. For spontaneous excitatory post-synaptic currents (sEPSCs), the holding potential was −70 mV and currents were pharmacologically isolated using bicuculline (10 μM; Sigma-Aldrich, NSW, Australia). A stable recording configuration was achieved for ~5 minutes before bath application of APV and CNQX or bicuculline to block sEPSCs or sIPSCs, respectively. Following the application of these antagonists, neurons were allowed to equilibrate for a minimum of 10 minutes. After this equilibration period, pindolol (10 μM, Sigma-Aldrich, NSW, Australia) was added to the bath. To determine the effect of pindolol on spontaneous synaptic activity, we compared the 2-minute epoch recorded from each cell prior to the application of pindolol with those obtained following 12–20 minutes of pindolol application. For experiments performed in the presence of the 5-$HT_{1A/1B}$ receptor antagonist methiothepin (Sigma-Aldrich, NSW, Australia), a final concentration of 5 μM was added to the Ringer solution and a minimum equilibration period of 15 minutes was included prior to the addition of pindolol.

## Drugs and chemicals

Pindolol [1-(1*H*-Indol-4-yloxy)-3-(isopropylamino)-2-propanol,1-(1*H*-Indol-4-yloxy)-3-[(1-methylethyl)amino]-2-propanol,Sigma-Aldrich, NSW, Australia] was dissolved in 2 percent dimethyl sulfoxide, 0.1 M HCl, 25 percent (2-Hydroxypropyl)-β-cyclodextrin solution (Sigma-Aldrich, NSW, Australia) and saline. The pH was adjusted to 7 using 0.1 M NaOH. (-)-CGP12177 was kindly donated by A/Prof. Peter Molenaar (Queensland University of Technology) and was dissolved in saline for systemic injections. Buspirone hydrochloride (N-{4-[4-(2-Pyrimidinyl)-1-piperazinyl]butyl}-8-azaspiro[4.5]decane-7,9-dione hydrochloride) (Sigma-Aldrich, NSW, Australia) was dissolved in saline. The 20 percent

ethanol (v/v) solution was prepared using 100 percent food grade ethyl alcohol (Recochem, SA, Australia) and filtered water. Sucrose (Chem supply, SA, Australia) solutions were prepared in filtered water.

### Statistics

All statistical analyses were performed using GraphPad Prism 6 (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using unpaired Student's *t*-tests or one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons post-test or a Fisher's LSD post-test. A *p*-value of $<0.05$ was considered significant. All values are expressed as the mean ± SEM.

# Results

## Effect of systemic injections of pindolol on short-term and long-term ethanol consumption

To examine the effect of pindolol on short-term and long-term ethanol consumption, we administered i.p. injection of pindolol or vehicle to mice consuming stable levels of 20 percent ethanol following 4 and 12 weeks of ethanol exposure. Following short-term drinking, pindolol had no effect on ethanol consumption after 30 minutes [$F(3, 30) = 2.283$, $p = 0.09$; Fig. 1a]. For long-term ethanol drinking mice, pindolol was administered in two cohorts of mice. We then compared the effect of each dose of pindolol against the pooled vehicle data from both cohorts, which were not significantly different ($p = 0.24$, unpaired Student's *t*-test). One-way ANOVA showed that pindolol had an overall main effect on consumption of 20 percent ethanol at 30 minutes [$F(4, 67) = 8.580$, $^{****}p < 0.0001$; Fig. 1b]. Bonferroni's post hoc analysis revealed that pindolol (8, 16 and 32 mg/kg) significantly reduced ethanol consumption as compared with the vehicle (8 mg/kg; $^{**}p = 0.006$, 16 mg/kg; $^{**}p = 0.005$, 32 mg/kg; $^{**}p = 0.001$). Pindolol had an overall main effect at the 2-hour interval in animals exposed to short-term ethanol treatment [$F(3, 33) = 3.423$, $p = 0.0284$; Figure S1a] but Bonferroni's *post hoc* analysis did not show an effect of treatment between the groups. Pindolol also had an overall main effect on ethanol consumption at 2 hours in animals following long-term ethanol exposure [$F(4, 67) = 3.925$, $p = 0.0064$; Figure S1b]. Bonferroni's post hoc analysis revealed that pindolol (32 mg/kg) significantly reduced ethanol consumption compared with the vehicle (32 mg/kg; $^{**}p = 0.001$). Pindolol had no effect on water consumption following short-term ethanol exposure; 30 mins [$F(3, 33) = 0.1651$, $p = 0.91$; Figure S3a] and 2 hours [$F(3, 33) = 0.4681$, $p = 0.70$; Figure S3b] but increased water intake following long-term ethanol exposure at 30 minutes (Figure S3c) and 2 hours (Figure S3d). Bonferroni's *post hoc* analysis revealed that at 30 minutes, pindolol (32 mg/kg) significantly increased water consumption as compared with the vehicle (32 mg/kg; $^{*}p = 0.01$) and at 2 hours pindolol (8 and 32 mg/kg) significantly increased water consumption (8 mg/kg; $^{*}p = 0.04$, 32 mg/kg; $^{*}p = 0.01$), suggesting that pindolol reduced ethanol consumption at these intervals but did not affect the total fluid intake of the animals. Similarly, pindolol had no effect on ethanol preference following short-term drinking (Figures S4a and S4b) but reduced ethanol preference following long-term consumption (Figures S4c and S4d). Bonferroni's *post hoc* analysis revealed that at 30 minutes pindolol (8 and 32 mg/kg) significantly reduced ethanol preference as compared with the vehicle (8 mg/kg; $^{*}p = 0.03$, 32 mg/kg; $^{*}p = 0.02$) and at 2 hours pindolol (32 mg/kg) significantly decreased the preference for ethanol (32 mg/kg; $^{**}p = 0.001$).

Effect of pindolol on 20 percent ethanol intake following short-term and long-term ethanol consumption. Pindolol had no effect on ethanol consumption following short-term exposure (A) but significantly decreased ethanol consumption following long-term exposure (B). Pindolol (8, 16 and 32 mg/kg, i.p.) or (4, 8, 16 and 32 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. Values are expressed as mean ethanol consumed (g/kg) ± SEM (one-way ANOVA followed by Bonferroni's *post hoc* test). $^{**}p < 0.01$ compared with vehicle ($n = 9–21$ per treatment)

## Effect of systemic injections of pindolol on short-term and long-term sucrose consumption

To comparatively assess the effect of pindolol on consumption of a natural reward, mice were given intermittent access to 5 percent sucrose for 4 or 12 weeks using the DID paradigm. Pindolol did not significantly alter the overall consumption of sucrose following short- [$F(3, 33) = 0.9137$, $p = 0.44$; Fig. 2a] or long-term exposure [$F(3, 31) = 1.002$, $p = 0.40$; Fig. 2b] at 30 minutes or short-term [$F(3, 33) = 0.2898$, $p = 0.83$; Figure S1a] or long-term exposure [$F(3, 31) = 0.4029$, $p = 0.75$; Figure S1b] at 2 hours. These results demonstrate a degree of specificity for pindolol to reduce consumption of ethanol but not the natural reward sucrose, following long-term intake.

Lack of effect of pindolol on 5 percent sucrose intake following short-term and long-term sucrose consumption, locomotor activity and LORR. Pindolol had no effect on 5 percent sucrose consumption following short (A) and long-term intake (B). Pindolol (8, 16 and 32 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. Values are expressed as mean sucrose consumed (g/kg) ± SEM (one-way ANOVA test, $n = 9–12$ per treatment). Pindolol also had no significant effect on ambulatory distances travelled compared with vehicle-treated naive (C) or long-term ethanol experienced mice (D). Values are expressed as mean distance travelled (m/5min) ± SEM (unpaired two-tailed Student's *t*-test). Pindolol also had no significant effect on the latency (E) and the duration of LORR (F) compared with vehicle-treated mice. Values are expressed as latency or duration (second) ± SEM (unpaired two-tailed Student's *t*-test, $n = 6$)

## Effects of pindolol on locomotor activity and LORR

To investigate any non-specific effects that may contribute to pindolol's ability to reduce ethanol intake, we examined its effects on general locomotor activity in naïve (18-week-old) animals. Pindolol (32 mg/kg) had no effect on locomotor activity in naïve animals ($p = 0.9558$, unpaired two-tailed Student's *t*-test; Fig. 2c) or long-term ethanol experienced animals ($p = 0.260$, unpaired two-tailed Student's *t*-test; Fig. 2b), suggesting that its ability to decrease ethanol consumption is not attributed to inhibition of locomotion of the animal. To examine if pindolol alters the sensitivity to ethanol we also performed the LORR test. Pindolol had no significant effect on the latency to LORR ($p = 0.6138$; Fig. 2e) or LORR duration ($p = 0.5121$; Fig. 2f) in the drug group as compared with the controls using a unpaired two-tailed Student's *t*-test ($n = 6$).

## Effect of systemic injections of (-)-CGP12177 and buspirone on long-term ethanol consumption

Because pindolol binds to both βARs and 5-$HT_{1A/1B}$ receptors, we investigated whether similar results could be obtained with compounds having activity at each receptor subfamily. We used (-)-CGP12177, which closely mimics the pharmacological activity of pindolol at βARs (Kaumann & Molenaar 2008) and the 5-$HT_{1A}$ partial agonist buspirone, which has similar affinity for 5-$HT_{1A}$ receptors compared with pindolol (Boess & Martin 1994). Our results show that the βAR drug (-)-CGP12177 had no overall significant effect on ethanol consumption [$F$ (3, 33) = 1.614, $p$ = 0.20; Fig. 3b] but did cause a trend towards a reduction in ethanol consumption at 80 mg/kg ($p$ = 0.105, repeated measures one-way ANOVA with Bonferroni *post hoc* test). In contrast, the 5-$HT_{1A}$ partial agonist buspirone significantly attenuated ethanol consumption in long-term drinking animals [$F$(3, 33) = 5.730, **$p$ = 0.002, repeated measures one-way ANOVA; Fig. 3a]. Bonferroni's *post hoc* analysis revealed that buspirone (2.5 and 5 mg/kg) significantly reduced ethanol consumption as compared with the vehicle (2.5 mg/kg; *$p$ = 0.04, 5 mg/kg; **$p$ = 0.001).

Effect of buspirone and (-)-CGP12177 on 20 percent ethanol intake following long-term ethanol consumption. The 5-$HT_{1A}$ partial agonist buspirone significantly decreased ethanol consumption following long-term exposure (A). The βAR compound (-)-CGP12177 did not decrease ethanol consumption, but did cause a trend to reduced ethanol intake at 80 mg/kg ($p$ = 0.105, B). Buspirone (1, 2.5 and 5 mg/kg, i.p.) and (-)-CGP12177 (vehicle, 30, 50 and 80 mg/kg, s.c.) were administered 30 minutes before the start of the drinking session. The values are expressed as mean ethanol consumed (g/kg) ± SEM (repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test; *$p$ < 0.05, **$p$ < 0.01 compared with vehicle, $n$ = 12 (buspirone) and $n$ = 9 [(-)-CGP12177] [Correction added on 27 June 2016 after the first online publication: Figure 3 caption has been revised.]

## Effect of buspirone on general locomotor activity and sucrose consumption

To determine non-specific activity, we examined the effect of buspirone on general locomotor behaviour and sucrose consumption. For locomotor testing, we administrated buspirone (2.5 or 5 mg/kg, i.p.) or vehicle in two different groups of 18-week-old naïve mice. Our results show that buspirone (2.5 mg/kg) had no effect on locomotor activity ($p$ = 0.63, unpaired two-tailed Student's $t$-test; Fig. 4b), while buspirone (5 mg/kg) significantly reduced locomotor activity as compared with the vehicle (**$p$ = 0.003, unpaired two-tailed Student's $t$-test; Fig. 4c). Additionally, repeated measures one-way ANOVA showed that buspirone significantly deceased sucrose consumption following long-term intake [$F$(3, 33) = 11.53, ****$p$ < 0.0001; Fig. 4a]. Bonferroni's post hoc analysis revealed that buspirone (2.5 mg/kg; *$p$ = 0.01 and 5 mg/kg; ****$p$ < 0.0001) significantly reduced sucrose consumption as compared with the vehicle.

Effect of buspirone on 5 percent sucrose intake following long-term sucrose consumption and general locomotor activity. The 5-$HT_{1A}$ partial agonist buspirone significantly decreased sucrose consumption following long-term exposure (A). Buspirone (1, 2.5 and 5 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. The values are expressed as mean sucrose consumed (g/kg) ± SEM (repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test; *$p$ < 0.05, ****$p$ < 0.0001 compared with vehicle, $n$ = 12). Buspirone (2.5 mg/kg) had no effect compared with vehicle-treated mice (B) but buspirone (5 mg/kg) significantly decreased ambulatory distances travelled (C). The values

are expressed as mean distance travelled (m/5min) ± SEM (unpaired two-tailed Student's *t*-test). $^{**}p < 0.01$ compared with vehicle, $n = 6$ over the 60-minute test period

## Effect of pindolol on spontaneous synaptic activity in BLA principal neurons

Because the BLA is widely implicated in drug seeking behaviour (Koob & Volkow 2010), and spontaneous synaptic activity in this region is altered by 5-HT and adrenergic drugs (Buffalari & Grace 2007; Rainnie, Asprodini, & Shinnick-Gallagher 1991; Wang, Cheng, & Gean 1999), we examined the properties of spontaneous synaptic currents recorded from BLA principal neurons in the absence and presence of pindolol using whole-cell voltage clamp recordings. In naïve mice, baseline characteristics of sIPSCs recorded from BLA principal cells did not change in the presence of pindolol (Table 1). In contrast, pindolol significantly decreased the sEPSC frequency (baseline vs pindolol, $p = {}^{**}0.0037$, one-way ANOVA followed by Fisher's LSD *post hoc* test; Table 1, Fig. 5a, d and g) of BLA principal cells without significantly changing sEPSC amplitude, rise time or half-width (Table 1). A representative electrophysiological recording, demonstrating the time-course of pindolol effect on sEPSC frequency is shown in Fig. 5g. The time-course of pindolol effect from all recorded BLA principal neurons is presented in Fig. 5a. Characteristics of sEPSCs in the absence and presence of pindolol are depicted in the representative traces of Fig. 5d.

Table 1. Electrophysiological properties of spontaneous post-synaptic currents recorded from BLA principal cells in the absence and presence of 10 μM pindolol.

| | Amplitude (pA) | Rise time (ms) | Half-width (ms) | Frequency (Hz) | n |
|---|---|---|---|---|---|
| Baseline (sIPSC) | 28.7 ± 3.8 | 3.5 ± 0.4 | 9.5 ± 1.3 | 2.1 ± 0.2 | 9 |
| Pindolol (sIPSC) | 29.5 ± 7.0 | 3.5 ± 0.5 | 9.4 ± 1.5 | 2.3 ± 0.3 | 9 |
| Baseline (sEPSC) | −13.5 ± 1.8 | 2.8 ± 0.3 | 3.2 ± 0.4 | 2.3 ± 0.3 | 8 |
| Pindolol (sEPSC) | −11.0 ± 0.7 | 3.0 ± 0.3 | 2.7 ± 0.2 | 1.4 ± 0.2** | 8 |

- All data presented as mean ± SEM. One-way ANOVA followed by Fisher's LSD *post hoc* test compared with baseline.
- ** $p < 0.01$.

Differences in the effect of pindolol effect on sEPSCs frequency in BLA principal cells from naïve and long-term ethanol-consuming mice. Time course of pindolol effect on sEPSC frequency from naïve (A) and long-term ethanol consuming mice in the absence (B) and presence (C) of the 5-$HT_{1A/1B}$ antagonist methiothepin (5 μM). Long-term ethanol consumption alters the effect of pindolol on sEPSC frequency from BLA principal neurons as shown in A and B. A significant increase in sEPSC frequency was observed in BLA principal cells from ethanol-drinking mice following 6 minutes of pindolol (10 μM) application (one-way ANOVA followed by Fisher's LSD *post hoc* test; $^{*}p = 0.015$ compared with baseline, $n = 8$). This effect was not present in naïve mice (A) and was blocked by the 5-$HT_{1A/1B}$ antagonist methiothepin (C). D to F shows representative traces of sEPSCs in the absence (top trace) and presence (bottom trace) of pindolol from naïve (D) and long-term ethanol drinking mice (E and F). Top trace in F was recorded in the presence of methiothepin. Calibration: 40 pA, 2 seconds. G shows representative time-course of pindolol effect on the

frequency of sEPSCs recorded from an individual neuron from naïve (circles) and long-term ethanol consuming mice in the absence (squares) and presence of methiothepin (triangles)

We then performed experiments in long-term ethanol consuming mice to determine the effect of pindolol on sEPSC properties. Comparison of basal BLA principal neuron sEPSC properties from naïve and long-term ethanol drinking animals showed no effect on amplitude (naïve: −12.2 ± 1.0 pA, $n = 8$; ethanol: −12.6 ± 0.6 pA, $n = 9$; $p = 0.70$, unpaired two-tailed Student's *t*-test) but did reveal a trend to reduced frequency (naïve: 1.8 ± 0.2 Hz, $n = 8$; ethanol: 1.4 ± 0.2 Hz, $n = 9$; $p = 0.10$, unpaired two-tailed Student's *t*-test). Interestingly, we also found that pindolol had a significantly different effect on sEPSC frequency from BLA principal cells in naïve and long-term ethanol drinking animals. We observed that prior to reducing sEPSC frequency, pindolol caused an increase in sEPSC frequency during the initial application period (Table 2, Fig. 5b, e and g). This effect was maximal at 6 minutes of steady-state application and was significantly different compared with baseline frequency (one-way ANOVA followed by Fisher's LSD *post hoc* test; * $p = 0.015$ compared with baseline, $n = 8$; Table 2 and Fig. 5b). Because pindolol binds to both βARs and 5-$HT_{1A/1B}$ receptors, we pre-incubated slices with 5 μM methiothepin for a minimum of 15 minutes prior to the addition of pindolol in order to block 5-$HT_{1A/1B}$ receptors. We then recorded sEPSCs in the presence of pindolol and found that bath pre-administration of methiothepin blocked the initial increase in sEPSC frequency caused by pindolol (Table 2, Fig. 5c and g). Furthermore, reductions in sEPSC frequency caused by pindolol were maintained in the presence of methiothepin (Table 2, Fig. 5c, f and g). These data suggest that changes in the effect of pindolol on sEPSC frequency in BLA principal neurons from naïve and long-term ethanol drinking mice are mediated via 5-$HT_{1A/1B}$ receptors.

Table 2. Electrophysiological properties of BLA principal cell spontaneous excitatory post-synaptic currents recorded from long-term ethanol consuming mice.

| | **Amplitude (pA)** | **Rise time (ms)** | **Half-width (ms)** | **Frequency (Hz)** | ***n*** |
|---|---|---|---|---|---|
| Baseline | −14.5 ± 0.9 | 2.9 ± 0.1 | 3.3 ± 0.4 | 2.6 ± 0.5 | 8 |
| Pindolol (6 minutes) | −14.6 ± 0.9 | 3.0 ± 0.2 | 3.5 ± 0.5 | 3.2 ± 0.6* | 8 |
| Pindolol (18 minutes) | −13.2 ± 0.8 | 3.4 ± 0.2* | 3.3 ± 0.3 | 2.1 ± 0.4* | 8 |
| Baseline with Met | −12.2 ± 0.8 | 3.1 ± 0.3 | 2.5 ± 0.2 | 1.7 ± 0.2 | 4 |
| Pindolol (6 minutes) | −12.4 ± 1.0 | 3.1 ± 0.2 | 2.7 ± 0.3 | 1.7 ± 0.2 | 4 |
| Pindolol (18 minutes) | −11.5 ± 0.9 | 3.0 ± 0.3 | 2.4 ± 0.2 | 1.2 ± 0.1* | 4 |

- Pindolol effects were determined in the absence and presence of 5 μM methiothepin. All data presented as mean ± SEM. One-way ANOVA followed by Fisher's LSD post hoc test compared with baseline.
- * $p < 0.05$.

# Discussion

Despite recent advancements that have identified key mechanisms and neural circuitry involved in the development of alcohol dependence, there remains a critical need for the development of improved treatment options for the management of AUDs. The DID

paradigm is a well-established model of binge ethanol drinking in mice, involving repeated ethanol exposure and abstinence, which promotes high levels of ethanol intake and pharmacologically relevant blood ethanol concentrations (Rhodes *et al.* 2005). Because repeated cycles of binge–drinking and abstinence are key components of the development of alcohol dependence, the DID protocol provides a useful high throughput approach for screening of pharmacological targets that can reduce binge patterns of ethanol consumption. Furthermore, chronic episodes of DID ethanol access (3–6 weeks) have been shown to promote increases in voluntary ethanol consumption following abstinence (Cox *et al.* 2013), an outcome that is also observed in other models of ethanol dependence (e.g. chronic intermittent ethanol vapor exposure) (Becker & Lopez 2004). These results combined with the growing body of evidence, which supports prolonged heavy alcohol consumption with the transition to dependence (Becker & Lopez 2004; Cox *et al.* 2013), suggest that potential drug candidates should have high efficacy in reducing alcohol consumption following long-term exposure. Consequently, we included short-term (4 weeks) and long-term (12 weeks) drug-testing time points in our experimental design. We report for the first time that pindolol, an FDA-approved drug for the treatment of hypertension, significantly attenuates ethanol consumption in long-term, high ethanol-consuming mice. Furthermore, we show that the efficacy of pindolol to reduce ethanol consumption is increased following long-term exposure, as compared with short-term (4 weeks) intake. We did observe differences in the efficacy of pindolol to reduce ethanol consumption at the 30-minute and 2-hour intervals. Because previous studies have shown that following a single oral dose of pindolol (5, 10 or 20 mg) in humans the peak in plasma concentration is reached within 1 to 2 hours (Jennings *et al.* 1979), pindolol's reduced effect on ethanol consumption at the 2-hour time point is most likely because of fast metabolism of the drug. Furthermore, because C57Bl6 mice consume more than half of their total ethanol intake in the 30 minutes (Rhodes *et al.* 2007; Wilcox *et al.* 2014), pindolol's robust activity during this period suggests that it effectively reduces the initial phase of binge-like ethanol consumption. Additionally, our results demonstrate selectivity for ethanol over a natural reward, no effects on locomotor activity and no effect on ethanol sensitivity. This suggests that the selective contribution of pindolol to reducing ethanol consumption following long-term intake is not because of non-specific effects.

GABA and dopamine signalling have been shown to be the immediate substrates of ethanol, and early adaptations in both signalling systems occur after acute exposure to promote tolerance (Harris, Brodie, & Dunwiddie 1992). As pindolol has no effect on short-term ethanol consumption, this indicates that the early phase of the reinforcing effects of ethanol are unaffected by systemic administration of pindolol. By contrast, our long-term drug testing data suggest that the expression and/or activity of βARs and/or 5-$HT_{1A/1B}$ receptors are altered following long-term binge-like ethanol drinking, leading to enhanced efficacy of pindolol to reduce ethanol consumption. Because pindolol has pharmacological activity at both βARs and 5-$HT_{1A/1B}$ receptors, we investigated the effect of compounds with activity at each receptor subfamily alone, following 12 weeks of ethanol drinking. Similar to pindolol, (-)-CGP12177 blocks $\beta_1$AR/$\beta_2$AR, but activates $\beta_{1L}$AR and $\beta_3$AR at higher concentrations (Kaumann & Molenaar 2008). Additionally, buspirone has similar affinity and potency for 5-$HT_{1A}$ receptors as compared with pindolol (Boess & Martin 1994). We show that (-)-CGP12177 (40 and 80 mg/kg) did not significantly disrupt ethanol consumption, while buspirone (2.5 and 5 mg/kg) reduced ethanol drinking following 12-week exposure. Buspirone (2.5 mg/kg) had no effect on locomotor activity, suggesting that it may specifically alter the rewarding properties of ethanol and sucrose to reduce their intake. However, buspirone significantly reduced locomotor activity at the highest dose (5 mg/kg), indicating a non-specific effect at this dose. Because buspirone has moderate activity at dopamine

receptors, the most likely explanation is that at higher doses, it might induce catalepsy by blocking postsynaptic striatal $D_2$ and $D_1$ receptors (Jadhav *et al.* 2008).

Compelling evidence is accumulating to suggest that both the NE and 5-HT systems play a critical role in alcohol seeking behaviour (Marcinkiewcz 2015). Interestingly, activation and partial activation of 5-$HT_{1A/1B}$ receptors have been shown to reduce ethanol intake in various experimental procedures (Crabbe *et al.* 1996; Sari 2013). Together with our results, this suggests that pindolol is most likely acting through partial activation of 5-$HT_{1A/1B}$ receptors. However, we cannot rule out the possibility that pindolol's combined effects at both βAR and 5-HT receptors contribute to its ability to reduce long-term ethanol consumption. Indeed, previous studies have shown that $\beta_1$AR/$\beta_2$AR (Gilpin & Koob 2010; Silberman *et al.* 2012) and $\beta_3$AR (Butler *et al.* 2014) compounds reduce ethanol seeking in dependent rodents. These studies showed that the non-selective $\beta_1$AR/$\beta_2$AR antagonist propranolol reduced responding for ethanol in dependent rats (Gilpin & Koob 2010). Furthermore, the $\beta_3$AR selective agonist BRL37344 was recently shown to reduce ethanol seeking in operant extinction trials (Butler *et al.* 2014). However, our results with (-)-CGP12177 did not produce significant reductions in long-term drinking. The lack of effect for (-)-CGP12177 may be due in part to its non-selective pharmacology, as (-)-CGP12177 possesses intrinsic sympathomimetic activity at $\beta_1$ARs by activating the low affinity site $\beta_{1L}$AR (Christ *et al.* 2011) and is also a partial agonist at $\beta_3$ARs (Kaumann & Molenaar 2008). Further investigations using more selective βAR and 5-$HT_{1A}$ receptor compounds are now needed to more precisely elucidate the pharmacological contribution of pindolol to reduce long-term ethanol drinking. This may uncover additional drug candidates that selectively reduce the consumption of ethanol following prolonged use.

To determine a possible site of action for pindolol and demonstrate its central effect in the amygdala, we assessed characteristics of spontaneous synaptic currents recorded from BLA principal cells in ethanol naïve and long-term exposed animals, in the absence and presence of pindolol. The BLA is a limbic brain region densely innervated by both serotonergic and noradrenergic fibres (Muller, Mascagni, & McDonald 2007; Zhang, Muller, & McDonald 2013). Furthermore, changes in BLA activity are widely implicated in alcohol dependence (Koob & Volkow 2010; Diaz *et al.* 2011). Our experiments show that in naïve animals, pindolol has no effect on sIPSCs, but causes significant reduction in sEPSC frequency in BLA principal neurons. These results are in line with previous reports demonstrating that 5-$HT_{1A}$ agonists reduced excitatory transmission in the BLA (Cheng, Wang, & Gean 1998; Rainnie 1999), while activation of βARs causes long-lasting enhancement of EPSCs in BLA neurons (Huang, Hsu, & Gean 1996). In addition, cross-talk between βARs and 5-$HT_{1A}$ receptors in the BLA has been demonstrated, with 5-$HT_{1A}$ receptor activation causing inhibition of isoproterenol-induced enhancements in synaptic transmission (Wang *et al.* 1999; Wang & Gean 1999). Interestingly, our results show that pindolol's activity in the BLA is altered following long-term exposure to ethanol and produces an increase in sEPSC frequency in principal neurons. We also show that this adaptive effect is blocked by methiothepin, a 5-$HT_{1A/1B}$ antagonist, which suggests that the increase in sEPSC frequency in BLA principal cells results from pindolol's partial agonist activity at 5-$HT_{1A/1B}$ receptors, presumably located in 5-HT boutons, which synapse onto glutamatergic terminals in the BLA. Additionally, these data implicate changes in 5-$HT_{1A/1B}$ signalling in the BLA following long-term chronic ethanol drinking. These results are also in line with previous studies showing an alteration of 5-$HT_{1A/1B}$ receptor function following chronic alcohol exposure (Burnett, Chandler, & Trantham-Davidson 2015; Kleven *et al.* 1995; Nevo *et al.* 1995). Thus, pindolol-induced partial activation of 5-$HT_{1B}$ inhibitory autoreceptors on 5-HT

terminals may reduce 5-HT release onto glutamatergic synapses, which may in turn, relieve the inhibition of glutamate release (Choi *et al.* 2012; Guo & Rainnie 2010) and increase the frequency of sEPSC in BLA principal neurons. In parallel, a 5-$HT_{1A}$ receptor-mediated inhibition of GABA release onto glutamatergic terminals (Lee *et al.* 2008; Schmitz, Empson, & Heinemann 1995) may also contribute to the observed increase in sEPSC frequency in BLA principal neurons. By sending direct glutamatergic afferents to dopaminergic varicosities (Johnson *et al.* 1994), the BLA positively modulates the dopamine efflux in the NAc (Howland, Taepavarapruk, & Phillips 2002) and deficiencies in accumbal dopamine have been observed in dependent animals after ethanol withdrawal (Weiss *et al.* 1996). Hence, in long-term ethanol exposed animals, pindolol may stimulate accumbal dopamine signalling via increased excitatory activity of BLA principal neurons to reduce their craving for ethanol. Similar results have been observed by our group with varenicline, a partial agonist of nicotinic receptors that reduces ethanol consumption and increases dopamine release in the NAc of long- but not short-term ethanol exposed animals (Feduccia *et al.* 2014). Although further studies aimed at uncovering pindolol's physiological mechanisms are required, the data reported in this study, combined with the well-established safety and tolerability profile of pindolol in humans (Gonasun & Langrall 1982), support the validity for further clinical development as a treatment for the management of AUDs.

The potential effectiveness of pindolol in the management of AUDs is also supported by studies that have demonstrated the contribution of βARs and 5-$HT_{1A}$ receptors to alcohol dependence in humans (Horwitz, Gottlieb, & Kraus 1989; Kraus *et al.* 1985). The βAR antagonist propranolol has been shown to reduce physical and motivational aspects of alcohol-seeking by reducing withdrawal tremors and stress in human alcoholics (Carlsson & Johansson 1971; Johnsson & Regardh 1976). Also, the βAR antagonist atenolol was shown to reduce alcohol craving in human dependents (Horwitz *et al.* 1989). Similarly, buspirone is more effective in treating alcohol-related psychopathological conditions and displays enhanced efficacy to reduce consumption in anxious alcoholic patients (Kranzler *et al.* 1994; Malcolm *et al.* 1992). Because pindolol has pharmacological activity at both βARs and 5-$HT_{1A}$ receptors, we propose that pindolol may more effectively reduce stress-induced anxiety and decrease ethanol craving and relapse as a result of a combined effect at these receptors, making it an attractive candidate that can be fast-tracked into human clinical trials.

In conclusion, our data show that pindolol, a FDA-approved drug having dual pharmacological activity on βARs and 5-$HT_{1A/1B}$ receptors significantly attenuates ethanol intake in mice following long- but not short-term ethanol consumption. Pindolol also shows selectivity for ethanol intake while having no effect on sucrose intake. Results from testing βAR and 5-$HT_{1A}$ selective compounds implicate changes in βAR/5-$HT_{1A}$ expression and/or signalling following long-term ethanol consumption that may enhance the efficacy of pindolol. Furthermore, we show that pindolol increases sEPSC frequency in BLA principal neurons from long-term ethanol consuming mice and this may increase BLA activity and facilitate reduced ethanol consumption following long-term intake. Although more research is required to further elucidate the mechanisms contributing to the effect of pindolol on long-term ethanol intake, we believe that pindolol represents a novel, safe and ready to test pharmacotherapeutic option for the management of alcohol dependence in humans.

[Correction added on 27 June 2016 after the first online publication: the ‘Discussion’ section has been revised.]

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All the authors that have participated in this study report no biomedical financial interests or potential conflicts of interest.

## Authors Contributions

PMK and SEB were responsible for the study concept and design. OLP carried out all behavioural animal experiments, data analysis and interpretation of findings. AB, JRT, JYH MRS and MM assisted in acquisition of data for the behavioural animal tests. PMK, MJF and AB performed the electrophysiological experiments and data analysis. PMK, OLP and AB drafted the manuscript. SEB, MCB and MJF provided critical interpretation of the data and the manuscript. All authors critically reviewed content and approved final version for submission.

Note:

The ‘General locomotor activity’ section of this manuscript was revised following a correction to the early online version was added on 27th June 2016.

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# Figures

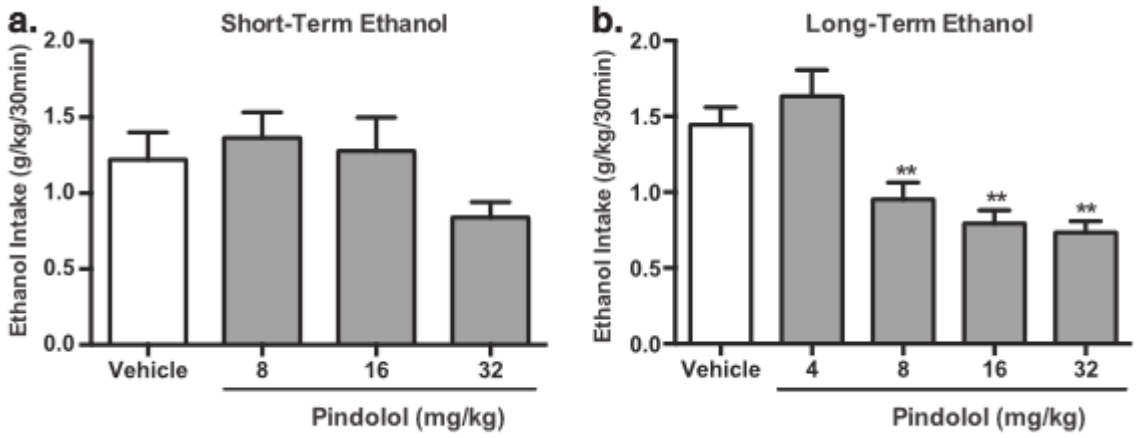


**Figure 1**

Effect of pindolol on 20 percent ethanol intake following short-term and long-term ethanol consumption. Pindolol had no effect on ethanol consumption following short-term exposure (A) but significantly decreased ethanol consumption following long-term exposure (B). Pindolol (8, 16 and 32 mg/kg, i.p.) or (4, 8, 16 and 32 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. Values are expressed as mean ethanol consumed (g/kg) ± SEM (one-way ANOVA followed by Bonferroni's post hoc test). **$p < 0.01$ compared with vehicle ($n = 9–21$ per treatment)

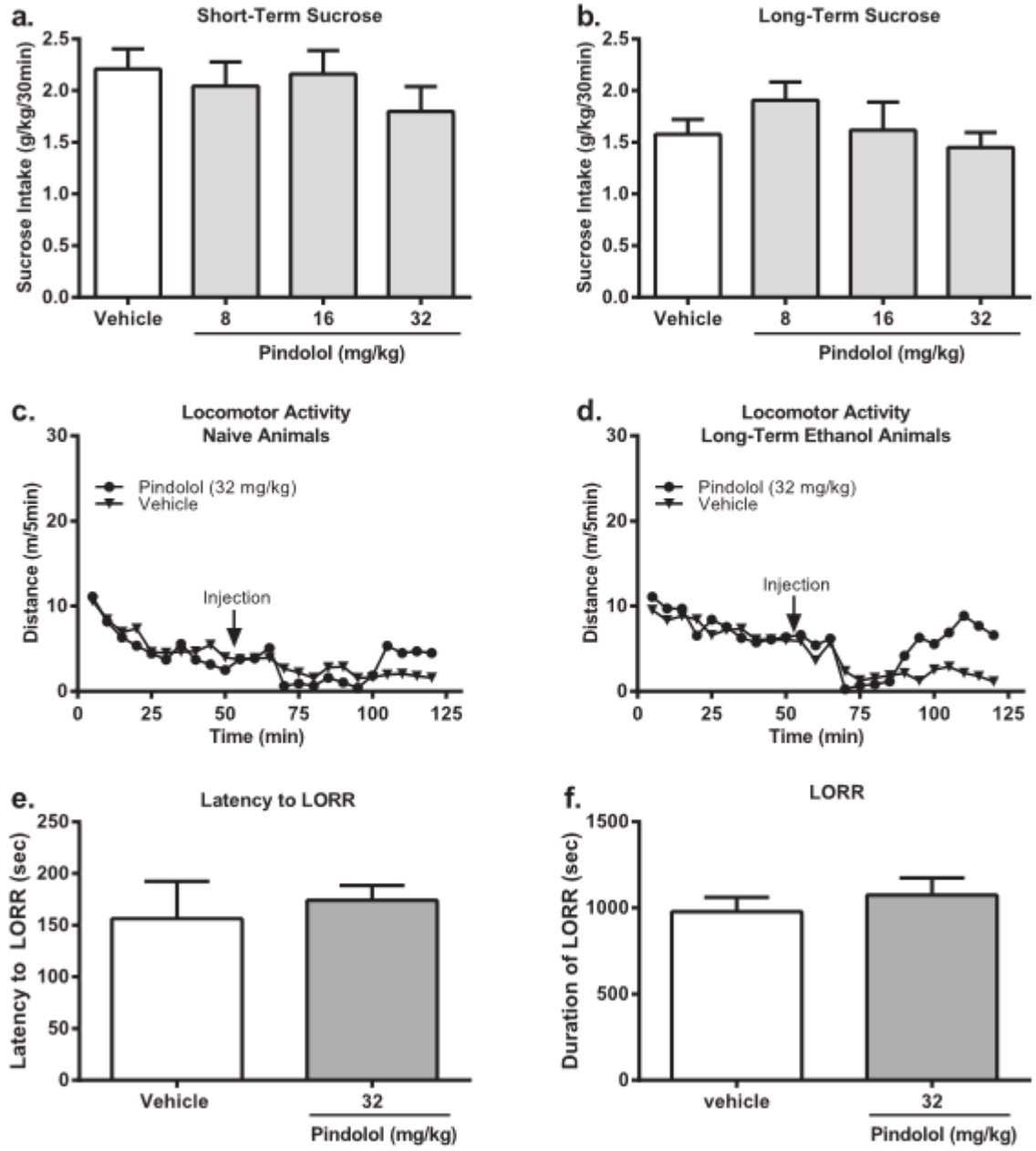


**Figure 2**

Lack of effect of pindolol on 5 percent sucrose intake following short-term and long-term sucrose consumption, locomotor activity and LORR. Pindolol had no effect on 5 percent sucrose consumption following short (A) and long-term intake (B). Pindolol (8, 16 and 32 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. Values are expressed as mean sucrose consumed (g/kg) ± SEM (one-way ANOVA test, $n = 9–12$ per treatment). Pindolol also had no significant effect on ambulatory distances travelled compared with vehicle-treated naive (C) or long-term ethanol experienced mice (D). Values are expressed as mean distance travelled (m/5min) ± SEM (unpaired two-tailed Student's *t*-test). Pindolol also had no significant effect on the latency (E) and the duration of LORR (F) compared with vehicle-treated mice. Values are expressed as latency or duration (second) ± SEM (unpaired two-tailed Student's *t*-test, $n = 6$)

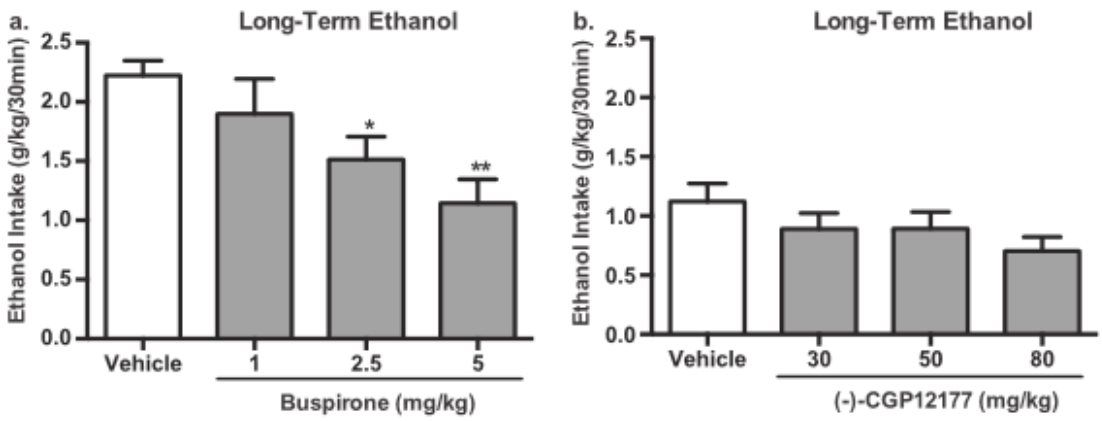


**Figure 3**

Effect of buspirone and (-)-CGP12177 on 20 percent ethanol intake following long-term ethanol consumption. The 5-$HT_{1A}$ partial agonist buspirone significantly decreased ethanol consumption following long-term exposure (A). The βAR compound (-)-CGP12177 did not decrease ethanol consumption, but did cause a trend to reduced ethanol intake at 80 mg/kg ($p = 0.105$, B). Buspirone (1, 2.5 and 5 mg/kg, i.p.) and (-)-CGP12177 (vehicle, 30, 50 and 80 mg/kg, s.c.) were administered 30 minutes before the start of the drinking session. The values are expressed as mean ethanol consumed (g/kg) ± SEM (repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test; *$p < 0.05$, **$p < 0.01$ compared with vehicle, $n = 12$ (buspirone) and $n = 9$ [(-)-CGP12177]

Note: manuscript caption revised after correction made to the first online version on 27 June 2016.

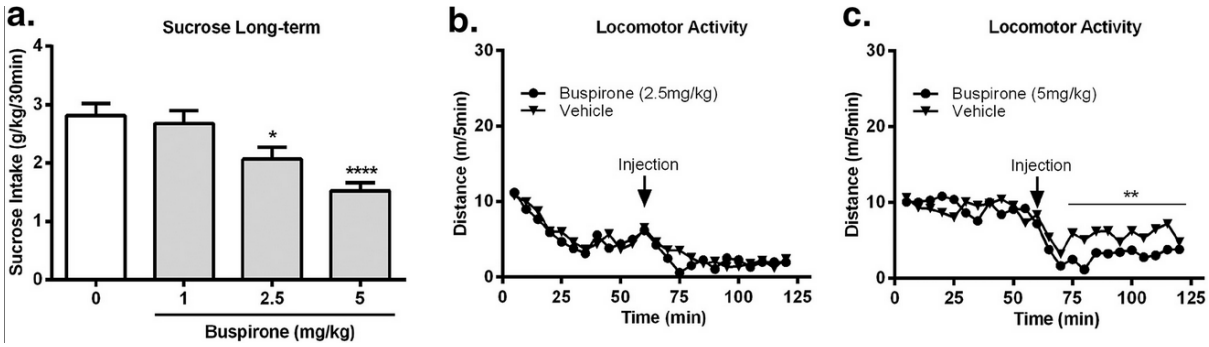


**Figure 4**

Effect of buspirone on 5 percent sucrose intake following long-term sucrose consumption and general locomotor activity. The 5-$HT_{1A}$ partial agonist buspirone significantly decreased sucrose consumption following long-term exposure (A). Buspirone (1, 2.5 and 5 mg/kg, i.p.) was administered 30 minutes before the start of the drinking session. The values are expressed as mean sucrose consumed (g/kg) ± SEM (repeated measures one-way ANOVA followed by Bonferroni's *post hoc* test; $*p < 0.05$, $****p < 0.0001$ compared with vehicle, $n = 12$). Buspirone (2.5 mg/kg) had no effect compared with vehicle-treated mice (B) but buspirone (5 mg/kg) significantly decreased ambulatory distances travelled (C). The values are expressed as mean distance travelled (m/5min) ± SEM (unpaired two-tailed Student's *t*-test). $**p < 0.01$ compared with vehicle, $n = 6$ over the 60-minute test period

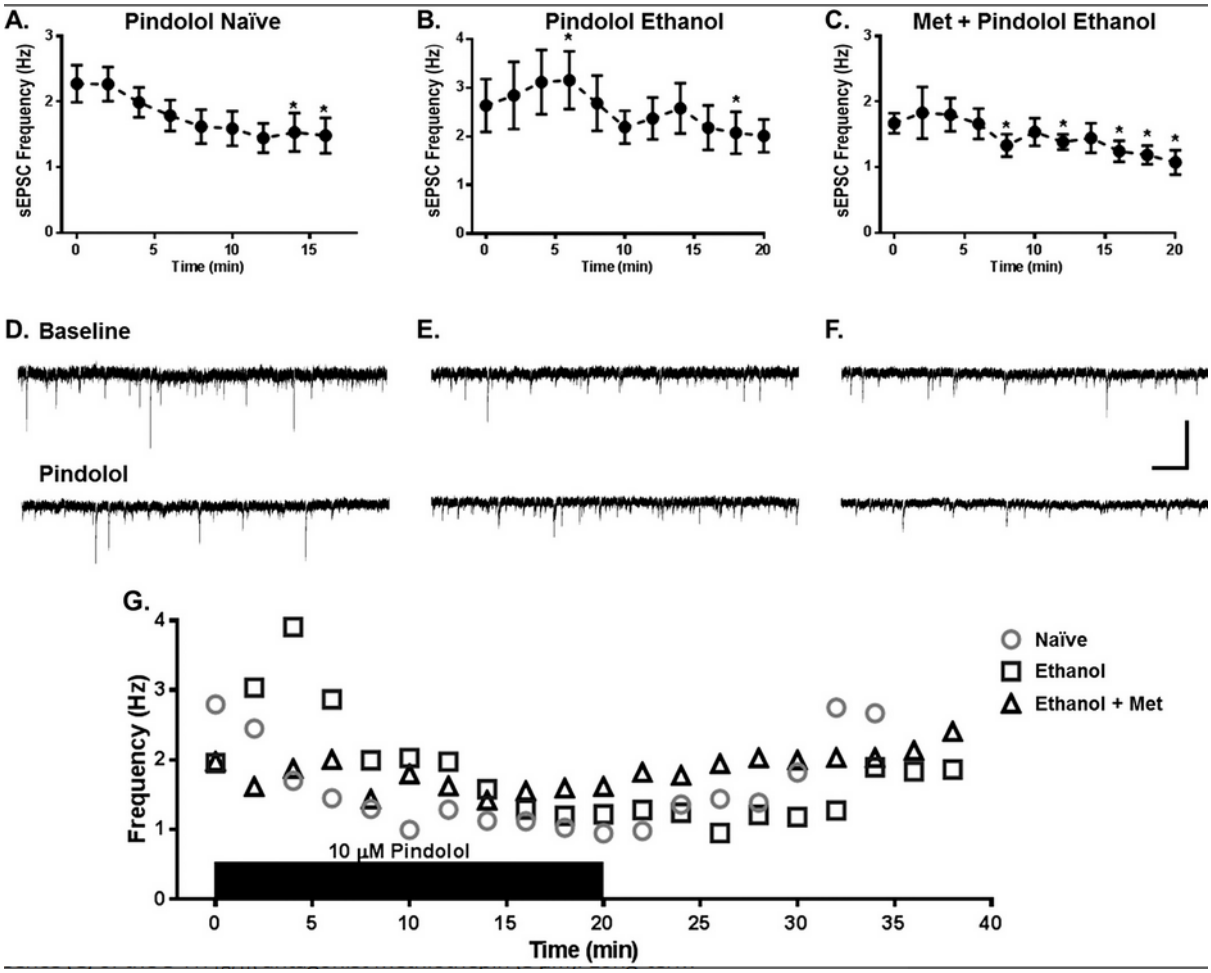


**Figure 5**

Differences in the effect of pindolol effect on sEPSCs frequency in BLA principal cells from naïve and long-term ethanol-consuming mice. Time course of pindolol effect on sEPSC frequency from naïve (A) and long-term ethanol consuming mice in the absence (B) and presence (C) of the 5-$HT_{1A/1B}$ antagonist methiothepin (5 μM). Long-term ethanol consumption alters the effect of pindolol on sEPSC frequency from BLA principal neurons as shown in A and B. A significant increase in sEPSC frequency was observed in BLA principal cells from ethanol-drinking mice following 6 minutes of pindolol (10 μM) application (one-way ANOVA followed by Fisher's LSD *post hoc* test; $*p = 0.015$ compared with baseline, $n = 8$). This effect was not present in naïve mice (A) and was blocked by the 5-$HT_{1A/1B}$ antagonist methiothepin (C). D to F shows representative traces of sEPSCs in the absence (top trace) and presence (bottom trace) of pindolol from naïve (D) and long-term ethanol drinking mice (E and F). Top trace in F was recorded in the presence of methiothepin. Calibration: 40 pA, 2 seconds. G shows representative time-course of pindolol effect on the frequency of sEPSCs recorded from an individual neuron from naïve (circles) and long-term ethanol consuming mice in the absence (squares) and presence of methiothepin (triangles)